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El secretoma de las células madre mesenquimales ¿Una alternativa válida para el tratamiento de las enfermedades hepáticas?

Mesenchymal stem cell secretome: a valid alternative for the treatment of liver diseases?

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Abstract

Chronic liver diseases represent a world-wide public health concern with a high prevalence and mortality rate. Although throughout the years a new therapeutic agent facing liver cirrhosis development has been researched, no one of them has obtained significant conclusions, remaining liver transplant the only curative therapy available.

Mesenchymal stem cells have gained increasing interest in regenerative medicine due to their important proliferative activity, their ability for self-renewal and the secretion of molecules with biological activities. They are a subtype of adult fibroblast-like cells which, under specifically defined in vitro conditions, could generate cells of neuro-ectodermal and endodermal origin. Due to the fact that many of their therapeutic effects can be attributed to their paracrine signaling, the administration of the mesenchymal stem cell secretome may preserve all the therapeutic actions avoiding the limitations derived from cell-based therapies, such as tumor formation, or cellular rejection.

The aim of this paper is to evaluate the scientific evidence on the possible application of the mesenchymal stem cell secretome as a potential acellular therapy in chronic liver diseases.

Resumen

Las enfermedades hepáticas crónicas son un problema de salud pública a nivel mundial, con una alta prevalencia y mortalidad. Aunque a lo largo de los años se han investigado nuevas posibilidades terapéuticas frente al desarrollo de la cirrosis hepática, ninguno de los estudios ha obtenido conclusiones significativas, siendo el trasplante de hígado la única terapia curativa disponible.

Las células madre mesenquimales han despertado un interés creciente en la medicina regenerativa debido a su importante actividad proliferativa, su capacidad de autorregeneración y la secreción de moléculas. Son un subtipo de células similares a los fibroblastos que, en condiciones in vitro definidas, pueden generar células de origen neuro-ectodérmico y endodérmico. Debido a que muchos de sus efectos terapéuticos pueden atribuirse a su señalización paracrina, la administración del secretoma de células madre mesenquimales puede mantener todas sus acciones terapéuticas evitando las limitaciones derivadas del uso de terapias celulares, como la formación de tumores, o el rechazo celular.

El objetivo de este trabajo es hacer una revisión bibliográfica sobre la actual evidencia científica en referencia a la posible aplicación del secretoma de células madre mesenquimales como potencial terapia acelular en enfermedades hepáticas crónicas.

1. LIVER FIBROSIS

1.1. GENERAL DESCRIPTION

Liver fibrosis is the result of chronic liver diseases (CLD) progression. Long-standing chronic parenchymal injury, of any kind, leads to a sustained activation of inflammatory response along with a constant activation of liver fibrogenesis and wound healing response. It is a dynamic and deeply integrated molecular, cellular and tissue process, responsible for the excess accumulation of extracellular matrix (ECM) components, which in the end is supported by the activation of hepatic myofibroblasts (MFs) (1).

MFs are the result of the trans-differentiation that hepatic stellate cells (HSCs) undergo, which are considered the main cell type that causes fibrogenesis (2). Different liver disease etiologies, such as toxic, metabolic, or viral, share common fibrogenesis pathways that involve hepatocyte damage and cause the infiltration of immune cells, activating this trans-differentiation (3). It has been recently described, that metabolic alterations in HSCs play an important role in this trans-differentiation process, offering more therapeutic intervention possibilities (2).

Physiologically, in short-term injuries, there is a counteracting process leading to an inactivation or apoptosis of myofibroblasts along with scar resolution. This mechanism is impaired in chronic liver diseases, in which an imbalance between pro-fibrogenic and anti-fibrogenic mechanisms is responsible of a persisting activation of proliferating, contractile, and migrating myofibroblasts, which produce an excess of ECM (3).

Although this liver response to injury is considered a “chronic wound healing reaction”, it actually entails the crucial factors of the progression towards liver cirrhosis, which is an advanced stage of CLD, defined by the development of regenerative nodules of parenchyma, fibrotic septa, and important changes in vasculature architecture. This condition can induce portal hypertension and derived complications such as variceal bleeding, hepatic encephalopathy, ascites, etc. Along with cirrhosis, the risk of developing hepatic failure and primary liver cancer is also increased (1).

1.2. EPIDEMIOLOGY AND ETIOLOGY

CLD is a complex condition associating a variety of clinical states, subcategories and overlapping etiologies. As it is shown in *Figure 1*, Europe has one of the broadest burden of liver disease in the world. CLD prevalence in this

continent varies widely depending on the area, etiology, ethnicity, gender or economic status, increasing slightly from Western to Eastern European countries with a higher incidence in Central European countries (4).

Underlying etiologies that lead to CLD are chronic infections (hepatitis B virus and hepatitis C virus), excessive alcohol consumption, non-alcoholic fatty liver disease, autoimmune liver diseases (primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH)), hereditary diseases (Wilson's disease, haemochromatosis and α1-anti-trypsin deficiency) (1). The contribution of these risk factors varies throughout the countries, explaining the variability on the prevalence and mortality of CLD. While alcohol is the most important risk factor in Western countries, viral hepatitis (B and C) has an important impact in Eastern countries, for instance (4).

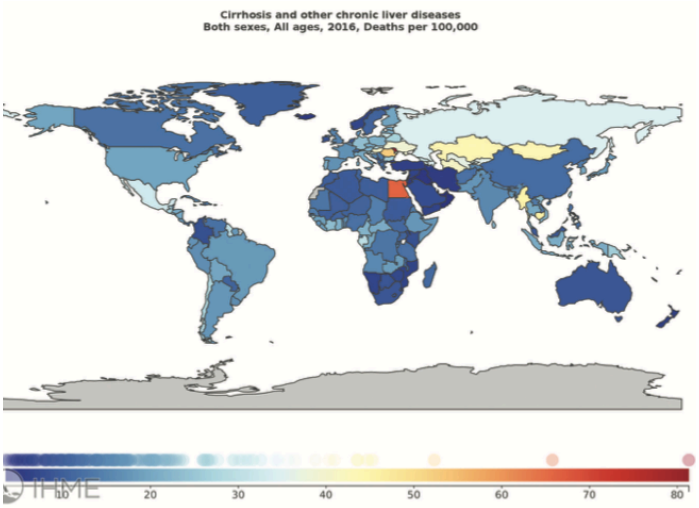

Figure 1. *Mortality from cirrhosis and other chronic liver diseases in 2016. Males and females all ages are represented (4).*

If we specifically focus in cirrhotic patients, prevalence ranges from 1.100 cases per 100.000 inhabitants in Austria or Romania to less than 450 cases per 100.000 inhabitants in Iceland (4). It is the main indication for liver transplantation (*Figure 2*), resulting in more than 5.000 cirrhotic liver transplantations per year in Europe (1).

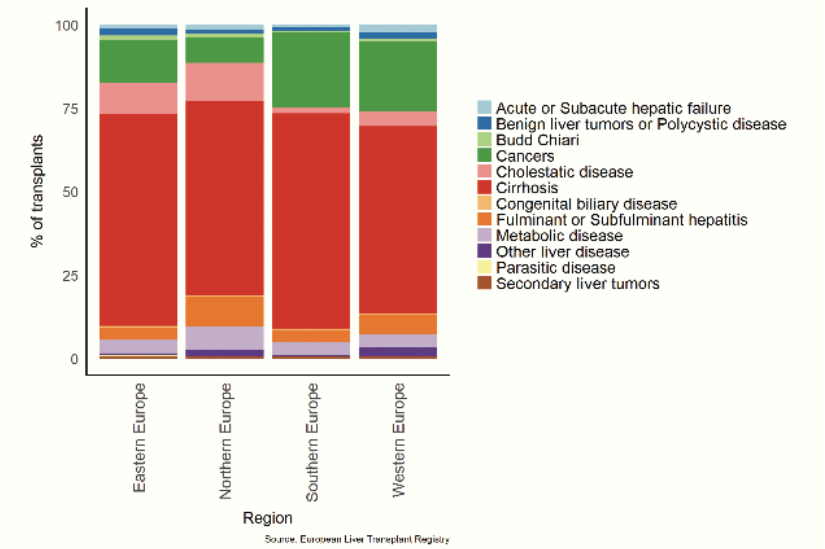

Figure 2. *Main indications of Liver Transplantation in Europe. 01/01/1968 – 30/06/2017 (4).*

Furthermore, cirrhotic livers have an increased risk to develop HCC, which is the 5th most common solid malignant tumor and the third cause of cancer-related death worldwide (1).

Altogether, it is important to highlight that liver fibrosis represents a world-wide concern, highly prevalent, with multiple etiologies and consequences, about which there is yet much to be discovered. In order to explain which mechanisms are involved in liver fibrosis development or could be used as a therapeutic target, a proper understanding of the mechanisms that lead to this end-stage liver disease is necessary.

1.3. PATHOGENESIS TOWARDS LIVER CIRRHOSIS

The term liver cirrhosis is defined by an advanced stage of CLD, described by the development of fibrotic septa which surround regenerative nodules of parenchyma, along with important changes in vascular architecture (1). It integrates a dynamic process, starting with parenchymal necrosis, activated fibrogenesis, followed by angiogenesis and profound vascular changes, in which chronic liver inflammation from any cause leads to liver cirrhosis. Mechanical obstruction and vasoconstriction enhances hepatic resistance to blood flow, progressively leading to portal hypertension, hyperdynamic circulation, bacterial translocation and increase of systemic inflammation (5). Throughout this development, the liver goes from a **compensated asymptomatic and potentially reversible state**, to a **decompensated symptomatic and irreversible state**, defined by the presence of portal hypertension and associated complications such as variceal bleeding, ascites, jaundice or

encephalopathy, for which nowadays there is only one therapeutic option: hepatic transplantation (1), (5).

An adequate vasculature in the liver is required due to its high metabolic activity, developing a complex structure of capillaries which are called the hepatic sinusoids (6). They are fenestrated capillaries, which represent a particular and unique blood vessel of the hepatic microcirculation. They stream, in the hepatic lobules, from the periphery up to central zone (7), converging in the centrolobulillar veins, which form the three suprahepatic veins, ending up in the inferior vena cava. During this streaming, most of the important liver functions are carried out (7).

In order to maintain liver function, the hepatic sinusoid is composed of specialized cells which communicate with each other. **Hepatocytes**, organized in hexagonal lobules, represent the parenchymal cells of the liver, separated from the sinusoidal endothelium by the space of Disse; **hepatic stellate cells**, located in this space, contribute to maintain sinusoidal tone and liver stiffness by releasing proinflammatory, anti-inflammatory cytokines and ECM components; **liver sinusoidal endothelial cells (LSECs),** which are unique endothelial cells due to the presence of open fenestrae and a characteristic lack of basement membrane (7), assemble hepatic sinusoids. LSECs display key functions in the maintenance of liver homeostasis, immune regulation and importantly, the modulation of the vascular tone and regulation of the coagulation cascade (8), and growth factors that enhance hepatocyte proliferation (9); **Kupffer cells (KC)** are the monocyte-derived macrophages residing in the liver. They are the immune cells which respond first to any damage in the liver. Liver injury exerts in all hepatic cells dysregulations which lead to phenotypic and functional changes (8). If damage persists, the liver will be driven into cirrhosis.

LSECs, suffer a *capillarization* process when liver is injured. This process is characterized by loss fenestrae, and development of a basement membrane. Blocking this “passage”, the necessary oxygenation of hepatocytes is diminished, causing severe injury. Hepatic injury, along with a reduced nutrition supply, drive hepatocytes to apoptosis and necrosis, and, lastly, the liberation of DAMPs (8).

LSECs protective properties are also affected, earning vasoconstrictor, pro-inflammatory and prothrombotic features(8), gradually leading to portal hypertension and hence, the following complications that define decompensated cirrhosis.

DAMPs and LSEC-derived factors directly activate HSCs and also produce recruitment and activation of lymphocytes and macrophages. They, by producing pro-inflammatory and pro-fibrogenic cytokines, actively participate in HSC trans-differentiation and myofibroblast activation (3). Kupffer cells acquire a

proinflammatory phenotype and liberate different cytokines which activate the immune response and inflammation process (8).

All in all, there is a cytokine-storm which induce signaling pathways, ending up in activating additional cells involved in fibrosis development, enhancing the pro-fibrogenic environment and maintaining the activation of HSCs and myofibroblasts (10). From a global perspective, Transforming Growth Factor ß (TFG-ß), Platelet Derived Growth Factor (PDGF), and the inflammasome (NLRP3)-Caspase1 pathway, along with WNT/ß-catenin signaling, are one of the main cytokines responsible of HSC activation and fibrosis development (3).

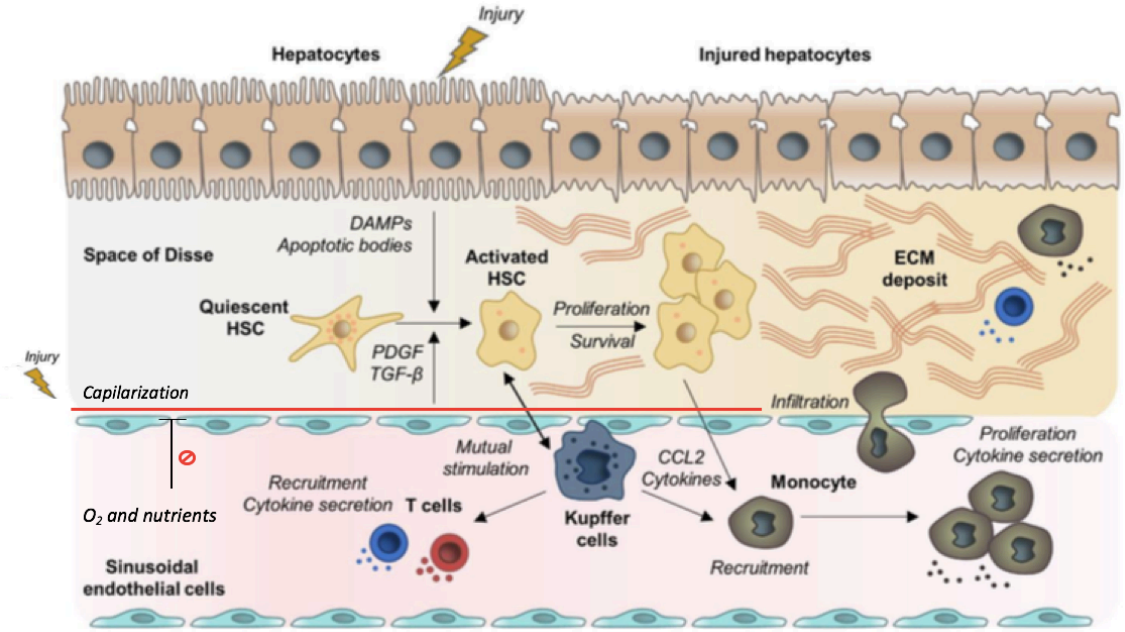

Figure 3. ***Mechanistic process for liver fibrosis*** (3). *Hepatic injury leads to hepatocyte apoptosis and secretion of damage-associated patterns (DAMPs) (3). When liver is injured, LSECs suffer a capillarization process, blocking the passage of oxygen and nutrients, and causing hepatocytes a severe injury. DAMPs and LSEC-derived factors directly activate HSCs and induce recruitment and activation of lymphocytes and macrophages, which also participate in HSCs trans-differentiation and myofibroblasts activation (3). Kupffer cells acquire a proinflammatory phenotype and liberate different cytokines which activate the immune response and inflammation process (8). In addition, there is a cytokine-storm inducing signaling pathways (TFG-ß, PDGF, inflammasome (NLRP3)-Caspase1 pathway, WNT/ß-catenin signaling) (3). Abbreviations: CCL2: chemokine ligand 2.*

1.3.1. HEPATIC STELLATE CELLS AND HEPATIC MYOFIBROBLASTS

MFs represent a heterogeneous population of highly proliferative and contractile alpha-Smooth Muscle Actin (alpha-SMA) - positive cells (1), which is an actin isoform correlated with activation of fibroblast into MFs, usually involved in inflammation, wound healing, fibrosis and carcinogenesis (11).

In normal conditions, MFs are not located in the liver, being their precise origin a matter of argument. Although MFs derive mostly from quiescent HSCs, through an activation/transdifferentiation process, additional sources have been recently described, such as portal track fibroblasts, circulating fibroblasts called fibrocytes, bone marrow derived cells and epithelial to mesenchymal transition (EMT) (12).

When HSCs remain continuously activated, they are able to coordinate incoming paracrine/autocrine signals. First, they are surrounded by a "profibrogenic environment" (Reactive oxygen species (ROS), growth factors, cytokines, chemokines, adipokines, proangiogenic mediators…). Second, hepatic cells (hepatocytes, KC, SEC, cholangiocytes, Hepatic Progenitor Cells (HPCs), resident lymphocytes) and extrahepatic cells (innate and adaptative immune cells which infiltrate and other bone marrow-derived cells) also liberate molecules responsible for CLD advance (1).

Hepatic Stellate Cells (HSCs), represent the 5-10% of the whole amount of cells in the liver, are the predominant cell type responsible for fibrotic process in the liver (2). They are star-like formed cells, quiescent and non-proliferative, with a large amount of cytoplasmic lipid droplets (3).

As it is shown in *Figure 4,* quiescent HSCs accumulate retinoids (vitamin A) in lipid droplets in the cytoplasm, which modulate tissue homeostasis and proliferation, differentiation and immune signaling, contributing to liver regeneration (2)(13).

The retinoid storage is lost after HSCs activation. These cells start to proliferate, contract, and enhance the synthesis of factors that contribute to the increase of ECM, such as pro-fibrogenic cytokines, growth factors, and morphogenetic proteins. The cellular cytoskeleton is altered due to the expression of α-SMA, modifying cellular motility and contraction, and regulating signaling processes during wound healing (2).

In short-term injuries, as damage decreases, HSCs are eliminated from the liver by apoptosis or deactivation (13). ECM is degraded by a number of

enzymatic families, being the matrix degrading metalloproteinases (MMPs) the main enzymes responsible for this process. They are a family of zinc and calcium dependent endopeptidases, secreted by connective tissue cells and inflammatory cells. Their targets are the major components of ECM, such as fibrillar and non-fibrillar collagens and elastin (14). As the elimination of the causal agent lacks in CLDs, HSCs deactivation is impaired (13).

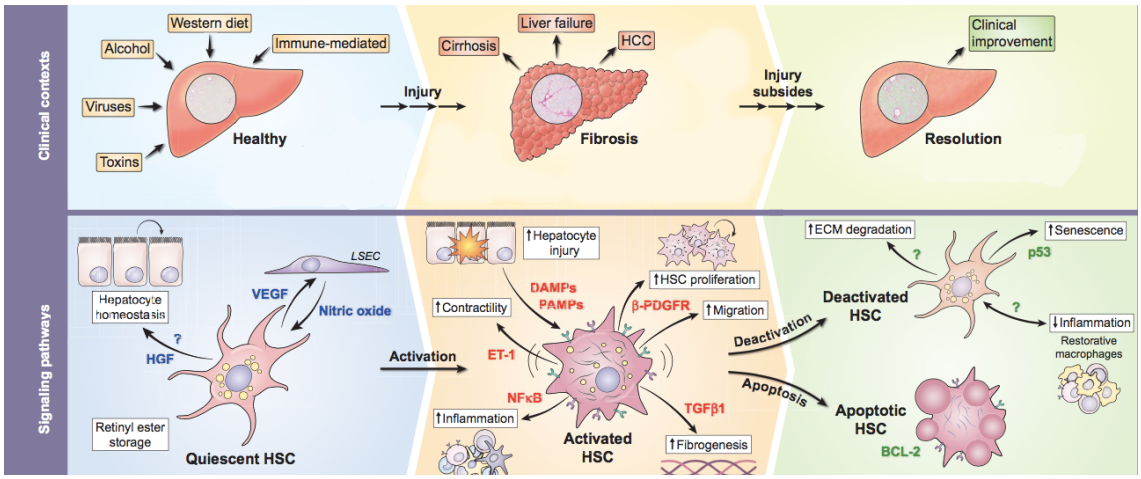

Figure 4. HCS trans-differentiation correlated with a clinical context (13)**.** *Liver damage produces wound repair and regeneration, along with ECM deposition. Fibrosis avoids progressively liver regeneration, enhancing the risk of developing liver failure. If liver injury disappears, hepatic fibrosis resolution can occur* (13)*. In a healthy liver, quiescent HSCs accumulate retinoids and lipid droplets in the cytoplasm, modulating tissue homeostasis (HGF) and proliferation, differentiation, immune signaling and intercellular communication with LSECs. During hepatic injury, the retinoid storage is lost and HSCs activate, proliferating, contracting and enhancing the synthesis of factors that contribute to the increase of ECM (2)*(13)*. From a chronic hepatic injury, pro- and anti-fibrogenic factors drive HSC into a clinical improvement through spontaneous resolution, or develop a maintained state with sustained pro-inflammatory and pro-fibrogenic microenvironment as well as liver ECM deposition (3). Abbreviations: HGF: hepatic growth factor.*

1.3.2. EPITHELIAL TO MESENCHYMAL TRANSITION

EMT cannot only be defined as a simple transition from the epithelial to mesenchymal state (15). Instead, it takes place when epithelial cells lose crucial epithelial hallmarks, such as apical-basal polarity, intercellular adhesion complexes, and adherence to a basement membrane, while becoming motile, invasive, and, sometimes, fibrogenic. EMT was recently divided into three categories: type I is related to development; type II with fibrosis, damage repair, and tissue regeneration; type III is mostly associated with cancer and metastasis. Mesenchymal cells are produced in type I, while type II produces fibroblasts that synthetize collagen, which can or cannot later develop into myofibroblasts (16).

Hepatocytes, HSCs and bile duct cells can become myofibroblasts through EMT and have an important role in the progression of liver fibrosis (15).

In the persistent inflammatory response in chronic injuries profibrotic mediators are secreted such as TGF-ß, contributing to the HSCs *transdifferentiation* process into MFs. TGF-ß is also responsible for an EMT process in hepatocytes, that may have an impact on the increase of the MFs population (17).

When EMT dictates over mesenchymal-epithelial transition (MET), fibrosis predominates in liver repair. However, when MET dominates over EMT, epithelial hyperplasia and fibrosis are diminished. This means that liver fibrosis development can be reduced or reversed through blocking EMT (15).

1.3.3. LIVER SINUSOIDAL ENDOTHELIAL CELLS

LSECs, phenotypically different from vascular endothelial cells, assemble the hepatic sinusoid. Due to the absence of basement membrane in the endothelium and existence of open fenestrations (6), an open communication between hepatocytes is facilitated. Furthermore, the entry of oxygen, micronutrients and macronutrients from the bloodstream is enabled (8).

In chronic liver diseases, these cells suffer a profound dedifferentiation losing their protective properties in injury, and becoming vasoconstrictive, proinflammatory and prothrombotic. After the *capillarization* process, the interchange of molecules such as lipoproteins and oxygen between hepatocytes is blocked, enhancing steatosis and parenchymal apoptosis. Through a decrease of Krüppel-like factor 2 (KLF2) and endothelial NO synthetase (eNOS) activity, Nitric oxide (NO) bioavailability is diminished, along with an increment of ROS, leading to HSCs activation and an increase of ECM (8).

They also release profibrogenic molecules such as TGF-ß, proinflammatory cytokines and respond to vasoactive substances such as endothelin, thromboxane A_2 (TXA2) inducing alterations in LSECs (8). Vascular endothelial growth factor's (VEGF) expression is also increased, inducing proangiogenic effect in LSECs and profibrogenic effect in HSCs (18).

This whole process increases vasoconstriction, leading consequently to microvascular dysfunction, fibrosis, and eventually portal hypertension (8), driving the cirrhotic liver into a decompensated and potentially irreversible stage with associated complications such as variceal bleeding, ascites, jaundice or encephalopathy.

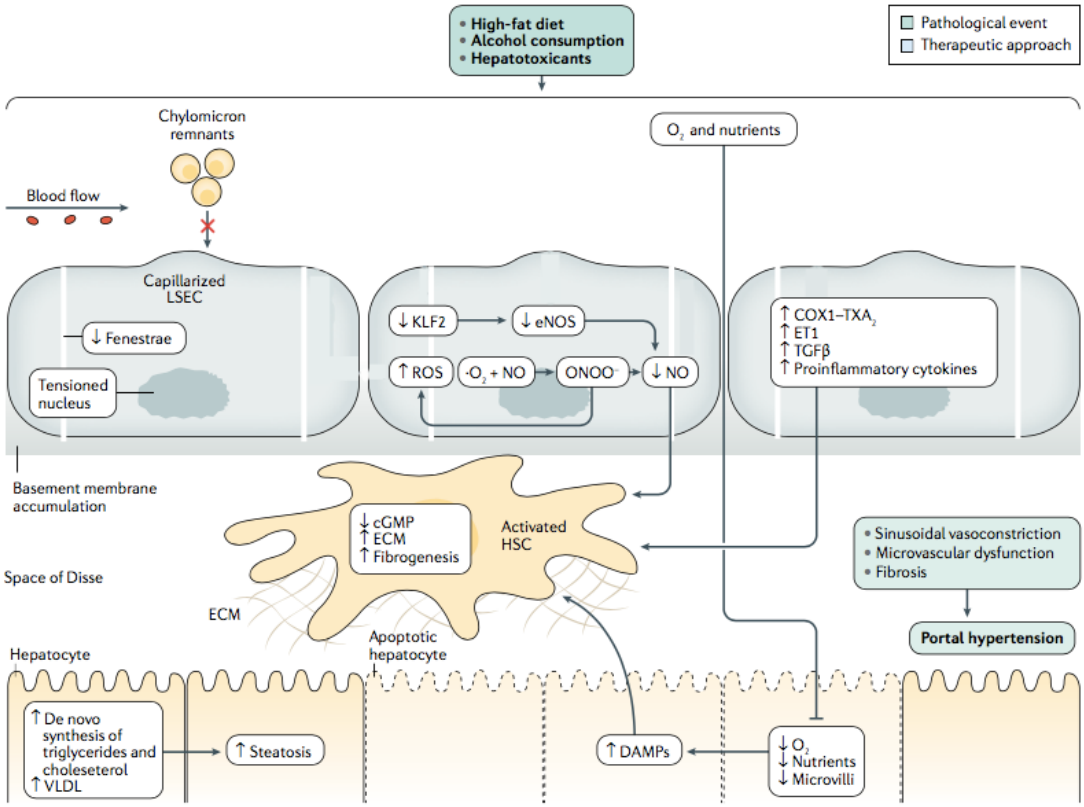

Figure 5. Pathobiology of LSECs in CLDs. *During liver injury, LSECs suffer a profound dedifferentiation, called capillarization, through which they lose fenestrae. This process blocks the passage of molecules and nutrients to hepatocytes, such as lipoproteins and oxygen, leading to steatosis and parenchymal apoptosis, releasing DAMPs. Through a decrease of Krüppel-like factor 2 (KLF2) and endothelial NO synthetase (eNOS) activity, Nitric oxide (NO) bioavailability in LSECs is reduced. They also release profibrogenic molecules such as TGF-ß, proinflammatory cytokines and respond to vasoactive substances such as endothelin, thromboxane A_2 (TXA2) inducing alterations in LSECs. The reduced NO bioavailability, the releasing of DAMPs and the release of profibrogenic molecules contribute to HSCs activation. This induces fibrosis, microvascular dysfunction and sinusoidal vasoconstriction, contributing to the development of portal hypertension (8).*

1.3.4. KUPFFER CELLS

KC are the resident macrophages in the liver, located in the hepatic sinusoid. This location allows them to efficiently phagocytize pathogens entering from the portal or arterial circulation, being considered as a final component of the gut barrier function. Kupffer cells thus play a major anti-inflammatory role, but a change in their normal functional activity can be observed under different disease states, contributing to liver fibrogenesis and inflammation, being the broadest non-parenchymal cells (NPCs) population in the liver (3). Kupffer cells display a remarkable plasticity depending on the metabolic and immune environment. Thus, they can express a range of polarized phenotypes that can be differentiated into two functional states, pro-inflammatory macrophages (M1)

and immunoregulatory macrophages (M2) (19). They are able to shift between them responding to a variety of stimuli, having an impact on fibrosis (10).

In liver injury, they are activated by a wide variety of DAMPs and pathogen-associated molecular patterns (PAMPs). This is triggered by the stimulation of the Toll-like receptor 4 complex (TLR4) by lipopolysaccharide (LPS), playing an important role in the increased immune response (20). Since blood leaving the gut empties directly into the portal vein, the liver is exposed to gut-derived endotoxin. As a result of endotoxemia, KC, are activated via the TLR-4 on the cell surface. This receptor is a member of the Toll-like family of pattern recognition receptors that are of central importance during host defense against invading pathogens. TLR-4 interaction with endotoxin results in the release of a myriad of pro-inflammatory mediators that induce hepatic injury and fibrosis such as interleukin-1ß (IL-1ß), IL-18 and many other pro-inflammatory cytokines and chemokines. This drives circulating leukocytes to be recruited and T cells to be modulated. Furthermore, it causes an up-regulation of vascular adhesion molecules on LSECs (1), and activation of HSCs (through the release of TGF-ß1) (18).

Activated macrophages, through the release of proline and arginase-1, increase collagen synthesis. A reduced fibrosis was demonstrated by inhibiting Kupffer cell function with gadolinium (12).

1.3.5. CYTOKINES AND PATHWAYS

A complicated and wide network of cytokines and pathways plays a key role in HSC activation and fibrogenic modifications (10).

PDGF is usually produced by platelets (10). It is a growth factor that induces HSCs division and proliferation (3). It is a molecule secreted by Kupffer cells, endothelial cells, and activated HSCs, which is induced to be released by variable etiologies of liver injury (10). After the binding of PDGF to its receptor, which is expressed at the membrane of HSCs (3), there is an activation of many signaling pathways, such as the PI3K/Akt, the JAK/STAT, and the Ras/Raf system. The expression of important pro-fibrotic genes, including type I collagen alpha1 chain, metalloproteinase (MMPs), tissue inhibitors of metalloproteinases (TIMPs) are regulated by these pathways. Furthermore, they also control apoptosis regulators (Bcl-1) that enhance survival and proliferation of MFs (10).

Endothelial cells, macrophages, and hepatocytes produce **TGF-ß** as a latent precursor, which accumulates in the ECM attached to the latency associated protein (LAP). A concrete protease cleaves this molecule, causing its activation. After the bound between activated-TGF-ß and TGF-ß receptor type II, TGF-ß receptor type I is recruited, causing a further activation of SMAD proteins.

Particularly, the central fibrogenic pathway is the activation of SMAD3, which increases fibrosis, through hepatocyte death and lipid accumulation. On the other hand, there are anti-fibrotic factors such as SMAD 6 and SMAD7, which down-regulate TGF-ß signaling. Moreover, IL-6 triggers the activation of STAT3. Its phosphorylation prompts the activation of the TGF-ß cascade. STAT3 up-regulates collagen mRNA expression in HSCs (10).

Wnt/ß-catenin signaling pathway represents another key pathway in liver fibrosis. ß-Catenin, an adhesion molecule, has also functions as a transcription factor (10), whose expression is regulated by the Wnt protein (3). After hepatic injury, the Wnt signaling pathway is triggered in the HSCs. Through an increase of alpha-SMA and collagen expression, this pathway plays an important role in fibrosis development (10).

1.4. LIVER FIBROSIS TREATMENT

The treatment of fibrosis aims four main aspects: elimination of the cause, suppression of inflammation in liver tissue, suppression of the activation of HSCs, and destruction of the extracellular matrix. Although the most effective way to prevent the formation of fibrosis is to eliminate its cause, it is not always possible (21).

As it has been previously described, there are many molecular pathways involved in the pathogenesis of liver fibrosis. Thus, targeting any of these pro-fibrotic pathways, liver fibrosis might be blocked or even reversed. Under this assumption, several targeted therapies have been developed in the recent years, but unfortunately, none of them has been proven to be effective in different randomized clinical trials (RCT) aiming at reversing liver fibrosis.

There are several clinical trials that exemplify this fact. First, Simtuzumab, a monoclocal antibody against lysyl oxidase-like 2 – which contributes to fibrogenesis by catalyzing cross-linkage of collagen, was ineffective in decreasing hepatic collagen content or hepatic venous pressure gradient (22). Las year, similar negative results have been communicated with other molecules, such as Selonsertib (a selective inhibitor of Apoptosis signal-regulating kinase 1 – which plays a key role in hepatocyte injury, inflammation and fibrosis in non-alcoholic steatohepatitis) (23), and Emricasam (an oral pan-caspase inhibitor) (24), which haven't been associated with regression of fibrosis.

At this current situation, there's neither a concrete treatment nor a path to follow. This opens a wide window of possibilities, being the use of Mesenchymal Stem Cells and its paracrine release of extracellular vesicles one of the new therapeutic approaches in fibrosis regression.

2. MESENCHYMAL STEM CELLS SECRETOME

In spite of liver tissue's outstanding ability to regenerate after injury, orthotopic liver transplantation (OLT) is still the only curative therapy in patients with end-stage liver disease or liver failure. Nevertheless, the resources are limited by donor insufficiency, the adverse effects of immunosuppressants, and ethical problems. Hepatocyte transplantation (HT), could be a plausible alternative to OLT, safer, uncomplicated and less invasive; however, there are also disadvantages in the use of HT, due to their limited proliferation capability, and the restricted liver functions of primary hepatocytes (9).

Fortunately, new therapeutic alternatives are emerging involving the use of mesenchymal stem cells (MSCs) from different origins and their paracrine effects, as candidates for liver regeneration in the context of liver diseases, focused on liver fibrosis.

2.1. MESENCHYMAL STEM CELLS

MSCs, a subtype of adult fibroblast-like cells, have an important proliferative capacity and the ability for self-renewal (25). They were first described about half a century ago, when bone marrow fibroblast-like colony-forming cells of mesenchymal origin were isolated and characterized. It was then shown that they could be induced to undergo differentiation into osteoblasts both *in vitro*, and *in vivo* (21).

Because of the great heterogeneity of MSCs from different tissues, the International Society for Cellular Therapy has defined three basic requirements that cells must meet to be considered MSCs. First, they must have a plastic-adherence in standard culture conditions; second, they must express the markers CD105, CD73, CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR in their surface; third, they must have the capacity to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (26).

The need of cells for regenerative medicine procedures has led to the search for new sources of MSCs. It has been shown that MSCs can derive not only from bone marrow, but also from perivascular cells from many other tissues, which include the liver (21). Thus, MSCs with similar *in vitro* properties have been successfully isolated from different tissues, such as synovial membrane, adipose tissue (AT), umbilical cord blood (UCB), amniotic fluid (AF) and placenta (25). It was thought that MSCs coming from different sources have indistinguishable or very close phenotype and characteristics. Nevertheless, contrary to the initial belief, there is increasing evidence of MSCs tissue specificity. Although the

reasons are not entirely understood, the assumption now is that MSCs treatments are most effective when treating diseases in the same tissue from which these cells derive (21). Umbilical cord tissue (UC) MSCs are an exception that makes them especially relevant as an MSCs source. UC-MSCs are believed to be more primitive cells compared to cells isolated from other sources, and are also present in larger numbers, which gives them increasing greater significance in regenerative therapies (25).

Under specifically defined *in vitro* conditions, MSCs could generate cells of neuro-ectodermal and endodermal origin, such as neuronal cells, hepatocytes, cardiomyocytes, alveolar and gut epithelial cells, finding themselves to be potential new therapeutic strategies in regenerative medicine (9), (27). The ability of MSCs to differentiate into hepatocytes suggested that regenerative therapies involving MSCs could be a promising therapeutic approach to liver injury and end-stage liver diseases (28). Accordingly, several studies demonstrated that the injection of MSCs mitigated fibrosis, reduced the hypoxic liver microenvironment, and increased liver functionality (10). As mentioned, MSCs have multilineage differentiation capability, which includes the transdifferentiation into hepatocyte-like cells. They can express liver-specific genes and show a mature hepatocyte function. However, some other properties of MSCs could explain their beneficial effect on liver pathologies (*figure 6*). MSCs can **modulate** both innate and adaptative **the immune response to injury** by releasing anti-inflammatory cytokines and factors, cell-cell contacts, and excretion of exosomes and microvesicles. They also secrete **trophic factors** to suppress activated HSCs and increase the proliferation of both resident hepatocytes and hepatic progenitor cells. They have an **hepatoprotective activity** by stimulating hepatocyte proliferation, inhibiting of hepatocyte apoptosis, producing an antioxidant effect and mitochondrial transfer. They also have an **anti-fibrotic impact** by regulating activated HSCs, reduction of pro-fibrogenic factors (TFG-ß), secretion of anti-fibrogenic factors (HGF, IL-10) and immune cells (21), (28). Remarkably, some published results demonstrated that, after treatment with MSCs, there was a reduction of TGF-ß1 and alpha-SMA gene expression in liver tissue (10). Moreover, they also release **extracellular vesicles (EVs)** which have a substantial therapeutic potential modulating liver injury (29). Both the ability to transdifferentiate and the secretion of molecules with biological activities give MSCs a unique therapeutic potential for regenerative medicine procedures (28).

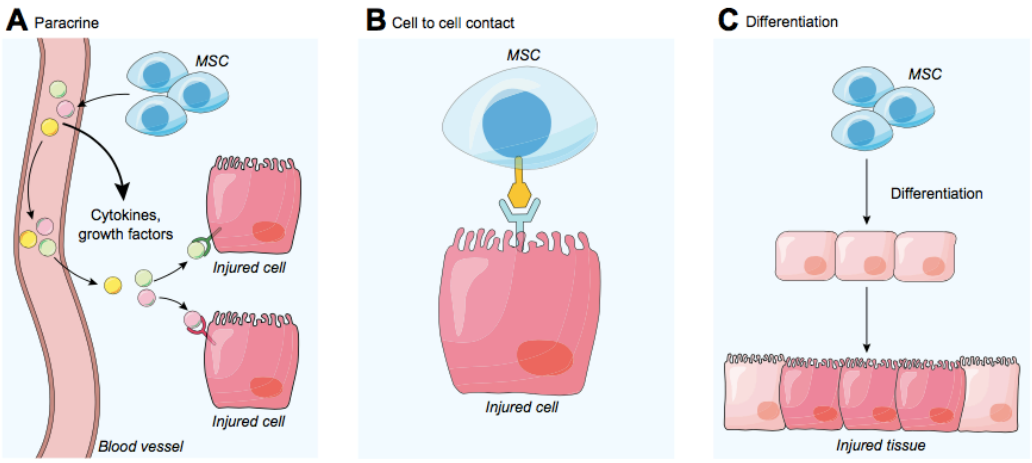

Figure 6. MSCs mechanisms of action (25)***.*** *MSCs exert their effects through a paracrine action (A), cell to cell contact (B) and multilineage differentiation (C). Many of the MSCs effects can be attributed to paracrine effects due to the secretion of cytokines, growth factors, and secretion of exosomes and microvesicles.*

Following liver injury, MSCs from different sources can be recruited to the liver (29). MSCs from different sources may have specific effects on liver fibrosis. Liver MSCs have an elongated and spindle-shaped form and they express not only classical mesenchymal markers, like vimentin and specific MSCs markers like CD990, but also several hepatic markers, indicating a higher hepatic commitment (21).

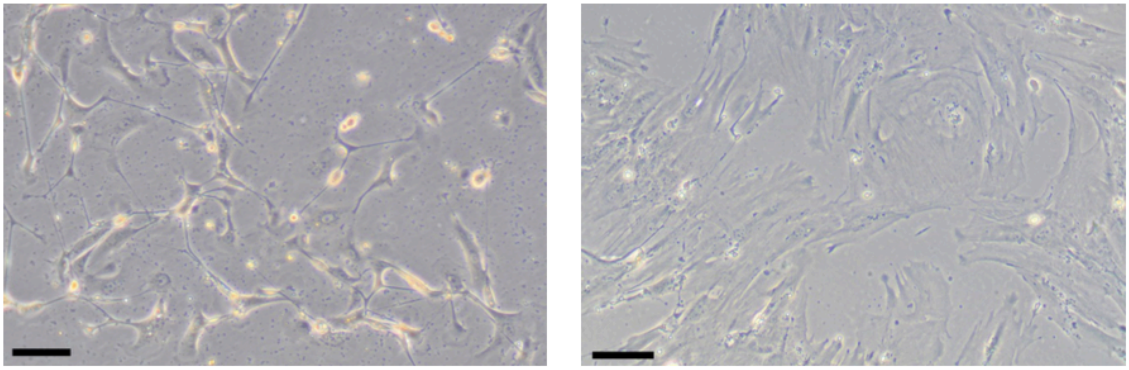

Figure 7. *Morphology of liver MSCs, derived from liver of patient with fibrosis. Bar scales: 25 μm* (21)*.*

Liver MSCs change their elongated morphology during hepatogenic differentiation, acquiring a polygonal shape. Furthermore, after hepatogenic differentiation *in vitro*, hepatocyte-like cells derived from liver MSCs show a down-regulation of mesenchymal markers expression, and an up-regulation on the expression of liver-specific genes and proteins, such as albumin, GATA4,

cytokeratins 18 and 19, cytochrome P450, alpha1-antirypsin, tryptophan 2,3-dioxygenase, and glutamine synthetase. After *in vitro* hepatogenic differentiation, liver MSCs behave to bona-fide hepatocytes, acquiring some of their functions such as *de novo* glucose production and urea synthesis, demonstrating the capacity of hepatic regeneration through differentiation (21).

Human umbilical cord (hUC-MSCs), and bone marrow MSCs (BM-MSCs), block the proliferation of HSCs, favoring their apoptosis, and thus, reduce the production of the main pro-fibrogenic factors (TGF-ß1), and induce de production of anti-fibrogenic cytokines such as Hepatic Growth Factor (HGF) and IL-10 by Ito cells (21). One of their targets is the TGF-ß1/SMAD's signaling pathway. In particular, they reduce SMAD3 expression and increase SMAD7, which can be regulated by different stimuli, including TGF-ß, IFN-gamma, and TNF-alpha. The downregulation of SMAD7 expression is related to both tissue fibrosis and inflammatory disease, and its over-expression antagonizes TGF-ß mediated fibrosis and inflammation (10).

After MSC transplantation, there is also a significant reduction of collagen deposition through the decrease in fibrogenic type I collagen alpha 1 gene expression. The reduction in pro-collagen gene expression often correlates with increased secretion of collagen-degrading MMPs (for example, MMP-9), which degrades ECM, and modulates genes involved in matrix remodeling (MMP-2 and TIMP-1) (10).

Mesenchymal stromal cells have also an indirect influence when it comes to fibrosis development through reduction of the hepatic inflammatory state. They inhibit proliferation of dendritic, T-helper-1, and natural killer cells; decrease production of pro-fibrotic TGF-ß in liver-infiltrated M1 macrophages, resulting in attenuated activation of HSCs and fibrosis reduction (27). They also induce activation of M2 macrophages, which secrete anti-fibrotic molecules (IL-10) and reduce deposition of collagen-1 in liver parenchyma. IL-10 may also modulate the expression of alpha-SMA, collagen I, and TGF-ß (10).

Despite all the benefits already described, cellular therapies have yet multiple limitations. One of the main concerns, limiting the use of MSCs-based therapy, is the differentiation into undesirable cell types or tumor formation (29). Furthermore, low engraftment or cellular rejection and their short half-life represent also important disadvantages. The injected cell dose, and the timing of treatment are some unsolved issues in the use of MSCs (10), (29).

Although several studies support the engraftment and correct differentiation of MSCs due to the increased expression of human hepatocyte substances, such as albumin and HepParl – a mitochondrial antigen of hepatocytes - some authors have also reported that, after *in vivo* injection, MSCs can differentiate into myofibroblasts increasing hepatic fibrosis, and consequently

contributing to fibrosis progression and avoiding liver regeneration. In fact, the expression of myofibroblast-like substances, such as alpha-SMA or glial fibrillary acidic protein, was seen in cells located around fibrotic areas after their administration (10).

There are several mechanisms through which MSCs's half-life is inadequate for tissue regeneration such as elimination by adaptative immune cells and the loss of their immune privileged status. Due to the expression of major histocompatibility complex class II as well as CD86, allogenic MSCs can lose their immune privileged status and be eliminated from the body. Moreover, allogenic MSCs, or transplanted autologous or allogenic MSCs could be eliminated by CD8+ cytotoxic T lymphocytes or by natural killer (NK) cells respectively (29). Due to the importance of their paracrine action, only administrating the secreted compound may avoid the negative effects related to cells, without diminishing their therapeutic potential.

Important issues related to management, such as cell dose, or timing of treatment are yet to be stablished. It is believed that the anti-fibrotic effect of MSCs is dose-dependent, due to a significant reduction in collagen release after using higher doses, compared to lower doses. Moreover, when MSCs are administered in earlier stages of liver disease, there is a more evident anti-fibrotic effect than when they are introduced after long-term injury, when beneficial effects are yet to be observed (10).

2.2. MSC-DERIVED SECRETOME

The short half-life, potential tumorigenic and difficult processing of MSCs, have prompted then the development of acellular therapies. Although MSCs are able to differentiate into a wide variety of cell types, thereby contributing to hepatic epithelial regeneration; many of their actions can be attributed to paracrine effects that occur due to the release of soluble proteins and EV secreted from the cells, which in the end constitute the MSC secretome (29). In fact, it was demonstrated in a large number of experimental studies that MSC-sourced secretome showed therapeutic effects similar to those observed after transplantation of MSCs (27). When the conditioned medium (CM) of MSCs was administered to mice after 70% hepatectomy, enhanced hepatocyte proliferation was observed as well as an up-regulation of genes such as TNF-alpha and HGF, and pro-angiogenic factor genes. This showed that, not only the cells, but also the CM, had an hepatoprotective action in liver fibrosis (21). Subsequent studies, an equally effectivity and safety in liver fibrosis between MSCs-derived secretome and MSCs (29), paving the way for the use of secretome for liver regeneration. The MSC-derived secretome is a complex mix containing all the active components of the CM. It is formed by a soluble component and extracellular vesicles (EVs) (*figure 8*). The active molecules of the soluble component are mostly proteins, such as cyto- and chemokines as well as growth factors and proteases (27).

EVs are a heterogeneous group of particles differing in size and composition. They are formed by a lipid bilayer membrane (29). Essentially all cells in our organism release them to de extracellular space, thus, can be obtained from all biological fluids. Their current classification depends on their size and biogenesis, finding exosomes, ectosomes and apoptotic bodies. Exosomes, ranging in size from 30 to 120 nm, are the EVs which are receiving the most attention. They originate through the inward invagination of the membrane of endosomal structures and are secreted by exocytosis, a process that consists on a fusion with the plasma membrane (10).

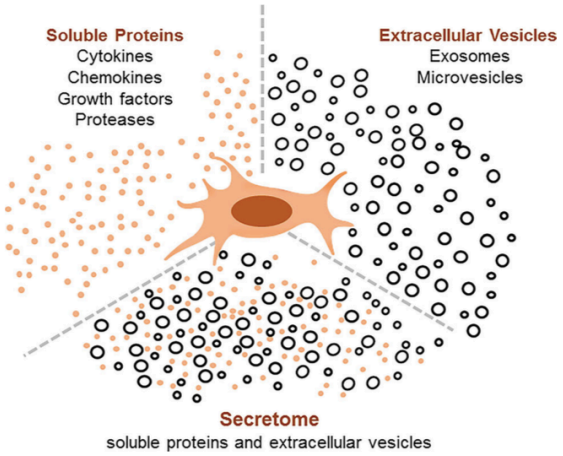

Figure 8. MSCs secretome. *It is composed of a soluble component (chytokines such as IL-10 and TNF-alpha, chemokines such as eotaxin-3, growth factors such as HGF and TGF-ß3, and proteases), and a non-soluble fraction, the extracellular vesicles including exosomes and microvesicles* (29).

Immune modulation, amelioration of injury and reduction of fibrosis are the main regenerative properties described in MSCs-derived secretome. Many MSC secretome components, soluble proteins as well as EVs, released by MSCs, contribute to these effects. They are both responsible of the therapeutic beneficial effects observed through their administration. Nevertheless, most studies reporting the effects of secretome are now focusing on EVs, isolated from the secretome (29).

An important role in liver fibrosis has been demonstrated through the administration of MSC-derived secretome. The different components have an impact on a variety of mechanisms that induce and maintain liver fibrosis, deteriorating hepatic function in many levels. When the etiology responsible for liver fibrosis cannot be eliminated, an advanced decompensated cirrhosis stage is eventually developed. The MSCs-derived secretome through antifibrotic, antiapoptotic and anti-inflammatory effects, and actions on HSCs and hepatocyte proliferation, could cause a decrease in the progression of the disease, as well as reverse the fibrotic development, slowing or stopping the progression towards a decompensated, and hence potentially irreversible, cirrhosis stage.

In fact, a study revealed that while an improve in chronic liver fibrosis through MSC-CM administration was seen, only a partial improve was demonstrated in hepatic failure. MSC-CM was administered in mice with hepatic

failure or chronic liver fibrosis. In spite of a noted therapeutic effect after 72h in mice with hepatic failure, survival did not improve significantly. This may demonstrate the enhanced liver repair system induced by MSC-CM, has an impact only at advances stages of self-recovery. On the other hand, an inhibition of collagen fiber accumulation, suppression of inflammatory infiltration and enhanced HSC apoptosis was seen in mice with chronic liver diseases after MSC-CM infusion (30).

Soluble proteins, such as cytokines and chemokines, contribute to immunomodulatory effects. They exert direct or indirect effects on different immune cells or on their responses to tissue or cell injury. There are also several growth factors and cytokines such as TGF-ß3, HGF, IL-10, and TNF-alpha, which modulate intercellular communication and processes involved in fibrogenesis, contributing to amelioration of liver fibrosis (29). HGF has a critical effect in hepatic regeneration. The release of this molecule into the parenchyma of damaged livers, is associated with a regression of liver fibrosis. It exerts anti-fibrotic effects by stimulating hepatocyte proliferation and inhibiting their apoptosis (10). In fact, blocking this molecule with antibodies, showed a reduction in the inhibitory effects of liver MSC-CM (21).

MSC-EVs show therapeutic effects in different preclinical models of hepatic fibrosis. EVs modulate different molecular pathways, exerting their effect on hepatocytes, activated HSCs and immune cells (10). In fact, they are able to modulate immune responses through the expression of surface markers, as well as specific tetraspannins such as CD63 and CD81 (29), which are part of multiple biological processes including cell adhesion, motility, invasion, membrane fusion, signaling and protein trafficking (31).

The possibility of modifying MSC-derived EVs cargo through biological engineering by adding several anti-fibrotic and anti-apoptotic proteins, or specific non-coding RNAs to include additional functions is becoming a very promising strategy as well as functionalizing the particles to direct them to specific target cells. No doubt these changes will son represent alternatives to today's EVs uses (29).

2.2.1. ANTI-INFLAMMATORY AND IMMUNOMODULATORY EFFECTS

Through modulating effector cells of both the innate and adaptative immune system, the MSC secretome has an immunosuppressive effect (29).

Immunomodulatory factors involved in the observed effects are TGF-β, HGF, indolamine 2,3-dioxygenase-1 (IDO-1), interleukin (IL)-10, IL-1 receptor antagonist (IL-1Ra) and prostaglandin E2 (PGE_2) (27). For example, PGE_2 and

IL-1Ra have an effect on macrophages, inducing their polarization into M2 phenotype which, through secretion of several molecules, such as IL10, can attenuate inflammation (29).

MSC secretion of chemokines, such as macrophage inflammatory protein (MIP), is able to recruit immune cells to the place of injury. Furthermore, the secretion of molecules such as NO and IDO responding to inflammatory stimuli, have also an effect on both innate and adaptative immune systems. IDO inhibits the proliferation of effector T cells, and increases CD4+CD25+ regulatory T cells (Tregs) expression (29). Through the secretion of TGF-ß, the IL-2-induced proliferation of CD4+T helper and cytotoxic CD8+T lymphocytes is diminished due to G1 ell cycle block (27). Due to Tregs secretion of IL-10, liver inflammation is attenuated (29). They also reduce toxic effects driven by natural killer T (NKT); by delivering thrombospondin 1 (TSP1), there is a suppression of TGF-ß/SMAD2/3 signaling, inhibiting proliferation and cytotoxic potential of NK cells (29), (27).

Embryonic stem cells (ESCs) are an alternative source of MSCs. ESCs-MSC-EVs showed immunomodulatory effects and stronger anti-inflammatory effects compared to other somatic tissue-derived MSC secretome. They have an immunomodulatory effect through diminishing immune cell infiltration, as well as regulating the expression of inflammatory cytokines through down-regulation of TNF-alpha and IL-2 levels, and up-regulation of TGF-ß and IL-10 levels (10).

Furthermore, hUC-MSCs-EVs have demonstrated a reduction in hepatic inflammation and collagen deposition. Molecularly, collagen I, III and TGF-ß expression were diminished after EVs administration (32). The amnion is another important source. In fact, an anti-inflammatory and anti-fibrotic effect was observed from EVs obtained from AF-MSCs. They reduced the expression of pro-inflammatory cytokines such as TNF-alpha, IL-1ß, IL-6, and MCP-1, and diminished the activation of pro-inflammatory M1 macrophages (Kupffer cells) in the liver (10), (32). Moreover, it was suggested that AF-MSC-EVs could inhibit the earlier steps of LPS/TLR4 pathway, and thus, reduce the release of proinflammatory cytokines (32).

MSCs secretome immunomodulatory effects can be previously modified by pre-conditioning MSCs before obtaining the secretome. A pre-condition of adipose stem cells (ASC) with lipopolysaccharide (LPS), could diminish the excretion of inflammatory cytokines such as TNF-alpha and IL-6 (10).

Moreover, IGF-1 containing UC-PVC-EVs, had an influence on hepatic macrophages through their conversion into anti-inflammatory phagocytes. The enhanced expression of aginase-1 and reduced expression of iNOS, TNF-alpha, and IL-6 contributed to this anti-inflammatory and immunomodulatory effect (10).

2.2.2. EFFECTS ON HEPATIC STELLATE CELLS

The MSC-derived secretome has also an anti-fibrotic effect on reducing the proliferation of activated HSCs and collagen synthesis (28), in fact, after treatment with serum EVs from healthy subjects, a significant inhibition of liver fibrosis and alpha-SMA down-regulation was induced, showing a diminished activation of HSC (32). This anti-fibrotic effect was demonstrated by a decreased ECM, together with a reduction in alpha-SMA-positive-HSCs and down-regulation of pro-fibrogenic genes (29). Molecules implicated in this function such as IL-10, HGF, TGF-ß3, and TNF-alpha, are able to inhibit HSCs proliferation and reduce collagen synthesis. Moreover, HGF and nerve growth factor (NGF) stimulate their apoptosis (28).

UC-MSCs secretome, decreased hepatic fibrosis both *in vivo* and *in vitro.* One of its components is milk factor globule EGF8 (MFGE8), an anti-fibrotic protein, the expression of which is reduced in fibrotic or cirrhotic livers. UC-MSCs diminished ECM deposition and inhibited activation of HSCs through downregulation of alpha-SMA expression and TGF-ß signaling pathway. A similar anti-fibrotic effect was observed in *in vitro* TGF-ß-activated HSCs, treated with CM coming from either AF-MSCs, or BM-MSCs (10). Moreover, treatment with BM-MSC-EVs in CCl_4-induced liver fibrosis, which is the most common hepatotoxin used to induce liver fibrosis in mice and rats (32), blocked the Wnt signaling pathway in activated HSCs through the downregulation of ß-catening, Wnt3s, Wnt10b, and PPAR-gamma (10).

The importance of miRNAs transported by EVs has been demonstrated in different experiments. On a rat model of CCl_4-induced liver fibrosis, it was shown that chorionic plate-MSC-EVs had the capacity of transferring miR-125b between MSCs and HSCs. If miR-125b is inhibited, a suppression of Hedgehog signaling is produced, diminishing consequently liver fibrosis (10). Besides, AT-MSCs-EVs from cells overexpressing miR-181-5p reduced liver injury and produced autophagy in HSCs through the inhibition of STAT3/Bcl-2/Beclin pathway. Furthermore, they down-regulated fibrosis-related genes, such as collagen I, vimentin, alpha-SMA, fibronectin and type I and III collagen, inhibiting HSCs activation induced by TGF-ß (10), (32).

Genetic engineering of EVs also plays an important role. In fact, an increased anti-fibrotic effect was demonstrated in EVs derived from UC-PVCs transduced by an adenovirus vector to produce human IGF-1. Treatment with IGF-1-containing UC-PVC-EVs, demonstrated an in vitro reduction of the activation of HSCs, by the downregulation of type I collagen, alpha-SMA and TGF-ß1 (10).

It was also demonstrated that overexpression of miR-122 with a lentiviral vector in AT-derived MSCs induced an increased efficacy against hepatic fibrosis

in AT-derived-MSC-EVs, compared to its non-transfected counterpart. AT-derived-MSC-EVs precisely transfer miR-122 to HSCs, blocking cell cycle and inhibiting miR-122-target genes CCNG1, IGF1R and PAHA1, by which proliferation and collagen maturation are regulated (10).

2.2.3. ANTIAPOPTOTIC EFFECTS

A significant reduction in hepatocyte apoptosis was demonstrated after treatment with MSC-CM, improving survival of D-gal-injured rats (29).

ESCs-MSC-EVs showed anti-apoptotic effects, as well as enhanced hepatocyte viability, and diminished the expression of pro-fibrotic molecules. In a murine TAA-induced chronic liver injury, a down-regulation of pro-fibrogenic factors such as collagen, alpha-SMA, and TIMP-1 was observed, while an increase of collagenases such as matrix metalloproteinase MMP-9 and 13 was also detected (10).

Engineering has also been effective when it comes to antiapoptotic effects. BM-MSC-EVs improved liver function in an autoimmune hepatitis murine model by down-regulating the expression of NLRP3 and caspase-1 genes related to the inflammasome and apoptosis. The anti-inflammatory and cytoprotective properties of BM-MSC-EVs were enhanced by lentivirus-induced upregulation of miR-223 in BM-MSCs, both *in vitro* and *in vivo*. Moreover, selective inhibition of miR-223 completely abolished the therapeutic effect of BM-MSC-EVs (10).

2.2.4. EFFECTS ON HEPATOCYTE PROLIFERATION

The effects of MSC-derived secretome on increasing cell proliferation and liver regeneration has been reported. Furthermore, numerous genes related to liver regeneration such as oncostatin M, adrenergic receptor-1 and stem cell factor were upregulated by CM treatment (29).

Murine hepatocytes following CCl_4 injury *in vitro* improved their viability, function activity, and proliferation, after being co-cultured with hUC-MSCs (29). *In vitro* hUC-MSC-EVs administration demonstrated a significant reduction of SMAD2 phosphorylation, which plays an important role in ETM-pathway, and inhibited the TGF-ß/SMAD signaling pathway through down-regulating type I and III collagen, and TGF-ß in hepatocytes (10), (32).

The treatment with MSCs secretome also demonstrated *in vivo* liver regeneration, following partial hepatectomy in mice. Administration of exosome-enriched BM-MSC secretome showed and enhanced liver regeneration in

partially hepatectomized rats with ischemia-reperfusion injury, which demonstrated that the vesicular fraction of the secretome may have a main role upon MSCs properties (29).

Pre-conditioning the MSCs before collecting the secretome can also intensify regenerative properties. Pre-conditioning ASC with LPS enhanced the regenerative response, and induced beneficial effects diminishing hepatic injury and increasing hepatocyte proliferation. In addition, hypoxia pre-conditioning of ASC led to a secretome product, that stimulated liver regeneration, showing an important effect in 1% partial pressure of oxygen (pO_2) exposure. In fact, following partial hepatectomy, hepatocyte proliferation was enhanced after treatment with hypoxia-primed ASC-CM. It was also related to a reduced suppression of cytokine signaling 3 (SOCS3) expression, and with an increased STAT3 signaling, HGF, VEGF, and Bcl-XL (29).

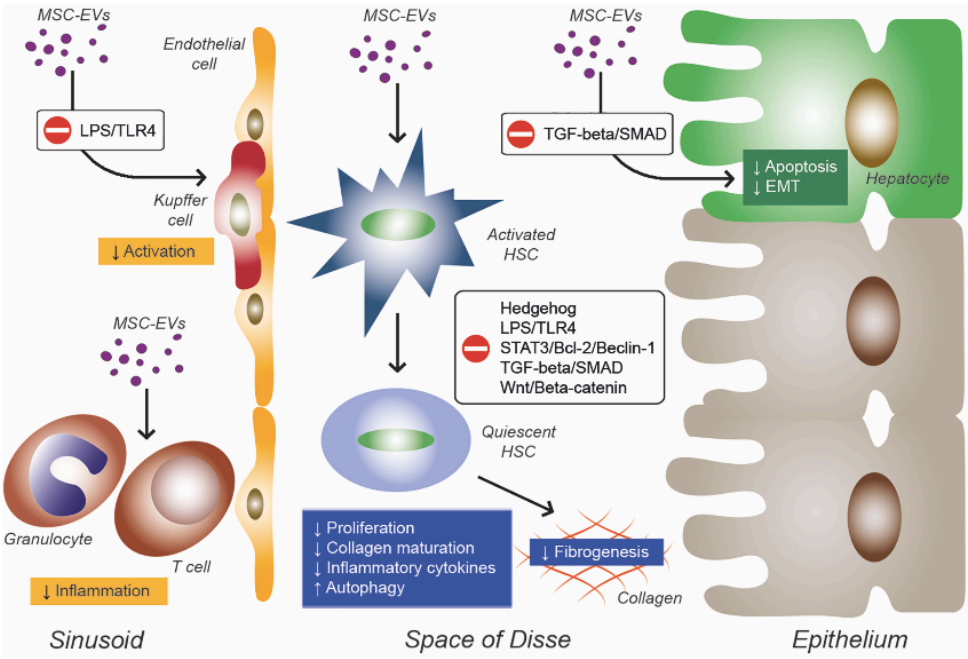

Figure 9. MSCs-derived secretome (10)***.*** *MSCs-derived secretome exert its actions through anti-inflammatory, immunomodulatory and antiapoptotic effects. They also have an impact on HSC and hepatocyte proliferation.*

2.3. PREPARATION OF MSC SECRETOME

The sequence for developing a therapeutic product based on MSC-secretome is schematized in *Figure 10*.

2.3.1. MSCs ISOLATION

MSCs isolation from different sources such as BM, AT, UC (33) and human liver MSCs is required. The parenchymal fraction of the liver is the source from which human liver MSCs are obtained, in most publications (21). The source from which MSCs are obtained could be relevant. It is demonstrated that MFGE8 is found in higher amounts in BM- and UC-MSCs, and it is enhanced after inducing these cells into hepatocyte differentiation. However, the embryonic MSC lack this factor (29).

The heterogeneity of MSCs sources, while introducing a complexity factor opens more possibilities. Through the study of the variety of cell-properties, behavior, state of differentiation, and inter-communication with their external milieu, we are provided with the opportunity to go towards designing functional applications based on the acknowledgment acquired after a detailed study of the therapeutic potential of each one of the different cell populations (29).

2.3.2. PRECONDITIONING OF MSCs

MSCs can be further modified in order to enhance desired functional effects. There are several processes through which beneficial alterations can be induced. Therapeutic molecules, such as mRNA, miRNAs and cytokines can be used. Furthermore, MSCs can undergo a pre-conditioning process which has demonstrated many favorable results (33). The pre-conditioning process can be carried out through serum starvation, hypoxia, pharmacological and physical stimulation, variations in matrix, culture in 3-dimensional cultures, the use of pro-inflammatory stimuli such as cytokines or LPS or the use of pro-differentiation stimuli (29), (33).

Hypoxic pre-conditioning increases regenerative and cytoprotective effects in MSCs. Its potential por maintain multipotency, increase proliferation and concentration of cytoprotective molecules has been demonstrated. MSCs are found in hypoxic environments *in vivo* (1% oxygen tension in cartilage and bone marrow, 12% in peripheral blood), which may explain this effect, responding favorably to hypoxia (34). The angiogenic potential of the secretome can also be improved through hypoxic pre-conditioning (29) due to an up-regulation of pro-angiogenic factors, such as VEGF and Angiotensin (34). It can also enhance the release of different immune mediators such as IL-10, IL-5, IL-6 and IL-13, as well as cytokines (29). Furthermore, it was demonstrated that the administration for hypoxia-pre-conditioned BM-MSCs, increased hepatocyte proliferation and survival and enhanced serum albumin levels (34).

Cytokine priming through the use of TNF-alpha and IFN-gamma down-regulates the secretion of cytokines (IL-10, IL-5, IL-6 and IL-13) and up-regulates the expression of cyclooxygenase 2 (COX2) (29).

The induced differentiation is another important pre-conditioning tool. UC-MSCs induced to differentiate into hepatocyte-like cells had an increased amount of glycoprotein, MFGE8, and an enhanced anti-fibrotic effect (29).

2.3.3. EXOSOMES ISOLATION AND PURIFICATION

Exosomes included in the MSC-CM, for which there is increased interest due to their easier production and storage and their smaller and less complex structure, are isolated, purified, characterized and quantified (33).

2.3.4. ADMINISTRATION

To target the liver, portal vein administration is an obvious choice (33). After infusion, a decreased efficiency is due to a fast clearance of EVs from the liver. Some modifications have shown increased bioavailability and thus ECS-MSC-EVs encapsulated in polyethylene glycol macromeres have revealed more efficient in a TAA rat model of hepatic fibrosis. Deliver into the peritoneum cavity can also be useful. Also, hydrogel-released EVs show stronger efficiency compared to freely injected EVs as shown by molecular and histological analysis. (10). Although most interest is now focused on MSC-EVs it has been increasingly proven that both EVs and the soluble proteins have beneficial effects through maintenance of tissue homeostasis in the place of injury (29).

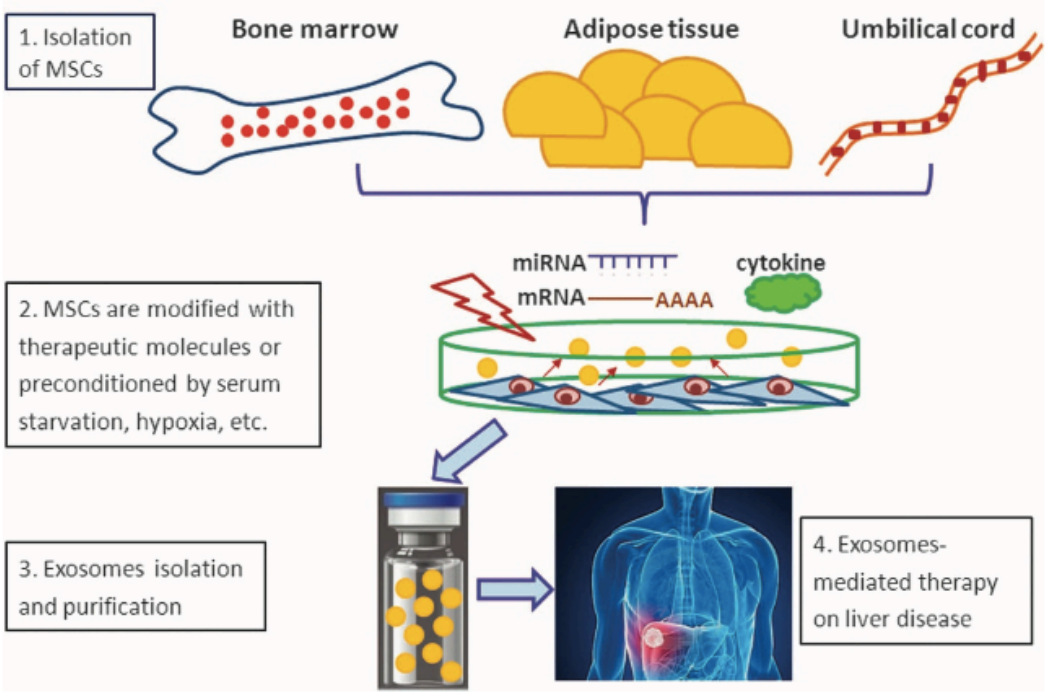

Figure 10. MSC-EVs obtaining process (33)***.*** *1. Isolation of MSCs; 2. Preconditioning of MSCs; 3. Isolation and purification; 4. Administration.*

2.4. UNSOLVED ISSUES IN EVs

Compared to cell-based therapy, EVs show stronger therapeutic efficacy. Furthermore, they can be engineered to carry a variety of molecules, such as RNAs, proteins and lipids, and tailored for specific functions like drug delivery (32). EVs are in fact a 'nature's delivery system' that could substitute another pharmaceutical drug vehicles, such as synthetic lipid and nanoparticles. Thus EVs are receiving increasing interest as a potential drug delivery option, which target cells through membrane fusion or endocytosis. Phagocytosis as well as degradation can be avoided, providing this 'nature's own cellular product' an advantage over other pharmaceutical drug vehicles, in fact, EVs can circulate for longer time periods (33).

Another concerning issue regarding MSCs was their short half-life. Compared to them, EVs have a higher stability and suitability for long term storage (32) remaining their effect potentially enough to obtain an effective therapeutic response.

Furthermore, it was demonstrated that problems related to cell rejection and tumor formation in MSCs, were diminished when using MSC-EVs. In patients who underwent allogenic administration of MSC-EVs, no adverse effects

regarding the immune system were reported (32). Moreover, there are no reports of oncogenic activity after MSC-EVs administration neither *in vivo* nor *in vitro*. In fact, they even have the potential to inhibit tumor growth, and an anti-tumor activity has been reported, through blocking cell cycle and inducing apoptosis and necrosis in a variety of cancer cell populations (32).

Although there are several advantages to the use of EVs, many other issues arise and need to be taken into consideration (32). Whereas a wide variety of MSCs sources has been described, and there is not clear which one is the best choice. hUC-MSCs may be one of the most potential cellular sources described, with an improved accessibility compared to BM-MSCs. Resident liver MSCs could be also a plausible cell source from which EVs can be obtained for the treatment of liver disease (32).

Furthermore, some practical issues such as the dose, the half-life or the quantity of EVs that need to be administrated in order to obtain a therapeutic response needs to be standardized (32). Also, the possible modification of EV's content possible modification deserves further attention, in particular the determination of which components have pro-regenerative or anti-fibrotic effects and which components may be harmful, as well as the possible negative effects derived from single or repeated administration (32). Thus, whereas research has shown many desirable and promising effects on liver fibrosis, there are unanswered questions that need to be studied in order to obtain an effective as well as safe acellular therapy as an alternative option to liver transplant.

3. CONCLUSIONS

MSCs secretome exerts its therapeutic effect on chronic liver diseases through the interaction of its soluble proteins and extracellular vesicles with the different mechanisms that induce and maintain liver fibrosis. They ameliorate the presence of HSCs, which are the cells with the greatest fibrotic impact in liver diseases, as well as reduce the immunogenic response that increases a proinflammatory and profibrogenic environment during liver injury. Furthermore, it reduces apoptosis and increases cell proliferation and liver regeneration.

Although it has not been yet entirely clarified, there is an assumption that a higher effectiveness can be reached when administrating MSCs derived from the same tissue which is targeted to be treated. However, many additional MSCs-secretome sources have been researched, showing different interesting properties that can be used for gaining benefits. Looking into the future, this only offers more advantages that may allow future therapies to be more effective and precise.

The administration MSC-EVs alone have gained increasing interest due to their easier manipulation, less complex structure and, their therapeutic potential.

Due to the development of different preconditioning strategies, and the fact that they can carry a variety of molecules which can be previously selected, a higher effectiveness can be reached. Nevertheless, there is a belief that adding both the soluble proteins and the EVs together may create a physiological effect, reaching a more effective and less harmful therapeutic strategy.

Although it has been demonstrated in experimental models that MSCs secretome may have an improve in chronic liver fibrosis, and not so much impact on hepatic failure, matters such as the effectiveness of the application on different stages of liver cirrhosis, or whether it has a potential therapeutic effect on decompensated cirrhotic livers need to be solved.

The fact that MSCs-derived secretome offers so many alternatives, implies a possible future development of therapies adjusted to the disease, or even the patient, although there is much yet to be discovered.

4. ABBREVIATIONS

α-SMA: Alpha-Smooth Muscle Actin

AF: amniotic fluid

AIH: autoimmune hepatitis

ASC: adipose stem cells

AT: adipose tissue

BM-MSCs: bone marrow stromal cells

CLD: chronic liver diseases

CM: conditioned medium

CREB: cyclic AMP-response element binding

DAMPs: damage-associated patterns

ECM: extracellular matrix

EMT: epithelial to mesenchymal transition

eNOS: endothelial NO sysnthetasa

ESCs: Embryonic stem cells

EV: extracelular vesicles

Exos: exosomes

HCC: hepatocellular carcinoma

HCSs: hepatic stellate cells

HGF: hepatic growth factor

HPCs: hepatic progenitor cells

HT: Hepatocyte transplantation

hUC-MSCs: Human umbilical cord mesenchymal stromal cells

IDO-1: indolamine 2,3-dioxygenase-1

IL: interleukin

IL-1ß: interleukin-1ß

IL-1Ra: interleukin 1 receptor antagonist

KC: Kupffer cells

KLF2: Krüppel-like factor 2

LAP: Latency associated protein

LPS: lipopolysaccharide

LSECs: Liver sinusoidal endothelial cells

MET: mesenchymal-epithelial transition

MFs: myofibroblasts

MFGE8: milk factor globule EGF8

MMPs: metalloproteinase

MSCs: mesenchymal stem cells

NGF: nerve growth factor

NK: Natural killer

NO: nitric oxide

NPCs: non-parenchymal cells

OLT: orthotopic liver transplantation

PAMPs: pathogen-associated molecular patterns

PBC: primary biliary cirrhosis

PDGF: Platelet Derived Growth Factor

PGE_2: prostaglandin E_2

pO2: partial pressure of oxygen

PSC: primary sclerosing cholangitis

RCT: randomized clinical trials

ROS: Reactive oxygen species

SECs: sinusoidal endothelial cells

TFG-ß: Transforming Growth Factor ß

TIMPs: Tissue inhibitors of metalloproteinases

TLRs: Toll-like receptors

TSP1: thrombospondin 1

TXA2: Thromboxane A_2

UC: umbilical cord

UCB: umbilical cord blood

VEGF: vascular endothelial growth factor

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